Journal of Applied Microbiology 2001, 90, 180–189

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Filter-based PNA *in situ* hybridization for rapid detection, identification and enumeration of specific micro-organisms

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414/6/00: received 19 June 2000, revised 2 October 2000 and accepted 11 October 2000

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Aims: A method for rapid and simultaneous detection, identification and enumeration of specific micro-organisms using Peptide Nucleic Acid (PNA) probes is presented.

Methods and Results: The method is based on a membrane filtration technique. The membrane filter was incubated for a short period of time. The microcolonies were analysed by *in situ* hybridization, using peroxidase-labelled PNA probes targeting a species-specific rRNA sequence, and visualized by a chemiluminescent reaction. Microcolonies were observed as small spots of light on film, thereby providing simultaneous detection, identification and enumeration. The method showed 95–100% correlation to standard plate counts along with definitive identification due to the specificity of the probe.

Conclusions: Using the same protocol, results were generated approximately three times faster than culture methods for Gram-positive and -negative bacterial species and yeast species.

Significance and Impact of the Study: The method is an improvement on the current membrane filtration technique, providing rapid determination of the level of specific pathogens, spoilage or indicator micro-organisms.

INTRODUCTION

Membrane filtration followed by growth on culture media to form visible colonies is a standard method for the detection of micro-organisms in filterable samples, such as water and beverages. In this way, low levels of micro-organisms are concentrated, and the number of colonies can be counted and related to the volume filtered. Dependent on the organism of interest, the origin of the sample and the growth media employed, filters are typically incubated for at least 18 h and up to several weeks before colonies are visible (Reasoner 1990). Except in rare cases where highly selective media exist, additional analysis to ascertain the identities of the micro-organism must be performed. Typically, one or more methodologies, including microscopic examination, biochemical analysis, or molecular technologies such as PCR or sequence analysis, are required.

The need for rapid detection, identification and enumeration of micro-organisms is extremely important in both environmental and industrial settings, particularly for non-sterile samples where a specific pathogen or indicator organism must be determined in the presence of indigenous organisms. Over the last decade, attempts to address this need have resulted in the development of rapid membrane filtration assays, using sensitive biochemical methods such as ATP-bioluminescence (Tanaka *et al.* 1997; Takahashi *et al.* 1999) and detection of esterase activity (Lisle *et al.* 1998), or molecular amplification methods such as PCR (Reysenbach *et al.* 1992; Sheridan *et al.* 1998). Although the use of biochemical methods provides both rapid detection and enumeration of micro-organisms, these techniques are not capable of identifying the micro-organisms on the membranes (Tanaka *et al.* 1997; Takahashi *et al.* 1999). On the other hand, molecular amplification methods like PCR can provide simultaneous detection and identification but lack the ability to enumerate. Furthermore, amplification methods are dependent on purified nucleic acids, and the high sensitivity of these methods often result in the detection of dead or non-culturable micro-organisms.

Ribosomal RNA molecules are key functional and structural elements of cells and are highly conserved between

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closely related species. Comparative study of rRNA sequences has allowed evolutionary microbiologists to define the phylogeny and taxonomy of bacteria, yeast and fungi (Fox *et al.* 1980; DeLong *et al.* 1989; Kurtzman and Robnett 1998). The next edition of *Bergey's Manual of Systematic Bacteriology* will reorder the phylogeny of bacteria based on these studies (Ludwig and Schleifer 1999). In addition, as rRNAs are present at a high copy number in bacteria, they have often been the target of choice for oligonucleotide probe assays (Amann *et al.* 1995). Recently, various groups noted a large variability in the degree of fluorescent signal dependent on the choice of the probe target region within the 16S rRNA molecule (Frischer *et al.* 1996; Fuchs *et al.* 1998). This variability was ascribed to poor accessibility of DNA probes to the rRNA targets because of the highly stable secondary structures in the rRNA (Amann *et al.* 1995; Frischer *et al.* 1996; Fuchs *et al.* 1998). Furthermore, DNA probes are often limited in their capability to distinguish single nucleotide changes. As a result, it is often difficult to design DNA probes that hybridize efficiently within a given stretch of the rRNA dictated by the nucleotide differences found between closely-related species.

Peptide nucleic acid (PNA) is a pseudo peptide that binds strongly and specifically to nucleic acids (Nielsen *et al.* 1991; Egholm *et al.* 1993; Buchardt *et al.* 1993). PNA consists of a non-charged polyamide backbone to which the different nucleobases are attached. The neutral PNA backbone confers unique properties on the molecule, relative to oligonucleotides. These include faster hybridization kinetics, a significant increase in Tm values for the PNA/NA complexes, and the ability to form a stable PNA/NA hybrid even at low salt concentrations. The latter point is important because the internal structure of dsDNA and rRNA is significantly destabilized at salt concentrations below 200 mmol l^{-1} (Perry-O'Keefe *et al.* 1996; Stefano and Hyldig-Nielsen 1997). Therefore, assay conditions can be chosen that favour the disruption of the target structure while still promoting strong hybridization of PNA (Stefano and Hyldig-Nielsen 1997). A similar approach is not possible for DNA probes as conditions that disrupt dsDNA and rRNA structures also adversely affect DNA binding.

A variety of membrane filter formats for bacterial detection and identification based on rRNA analysis have previously been described. These include assay methods employing directly, as well as indirectly labelled probes. In one method, visible colonies were identified following cell lysis and attachment of the released rRNA to the filter surface using alkaline phosphatase-conjugated DNA probes (Wright *et al.* 1993; Glover and Harris 1998). Prescott and Fricker (1999) described a filter-based multilayer assay PNA for *in situ* detection of *Escherichia coli* in water by microscopy, and Mittelman *et al.* (1997) used membrane filters to collect fixed bacteria from urine and then transferred the bacteria from the filter to a microscope slide for further hybridization and microscopic analysis. The use of microscopy to view bacteria requires a high cell density on the membrane surface, as only a small area of the filter/slide can be examined in a reasonable period of time.

In this paper, a filter-based *in situ* hybridization assay using peroxidase-labelled PNA probes is described. Filtration was used to concentrate, isolate and separate individual micro-organisms onto a membrane filter. The membrane was then placed on a culture medium and incubated appropriately according to the organisms of interest. Long before colonies were visible, the incubation was terminated and hybridization was performed with a peroxidase-labelled PNA probe complementary to a species-specific rRNA sequence. Hybridized probe was detected using a chemiluminescent reaction and exposure to X-ray or instant film. Microcolonies were observed as small dots on the film, thereby providing a rapid and simultaneous detection, identification and enumeration of the specific micro-organisms.

MATERIALS AND METHODS

Bacteria and yeast strains

The bacteria and yeast strains used in this study were obtained from either the American Type Cell Culture (ATCC), Manassas, VA, or the Agricultural Research Service Culture Collection (NRRL) Peoria, IL, as indicated: *Pseudomonas fluorescens* (ATCC 13525), *Pseudomonas aeruginosa* (ATCC 27853), *Burkholderia cepacia* (ATCC 25416), *Pseudomonas putida* (ATCC 12633), *Eschericia coli* (ATCC 25922), *Lactobacillus brevis* (ATCC 14869), *Staphylococcus epidermidis* (ATCC 14990), *Staphylococcus aureus* (ATCC 6538), *Salmonella choleraesuis* (ATCC 29946), *Dekkera bruxellensis* (NRRL Y-12961), *Dekkera anomala* (NRRL Y-17522), *Brettanomyces naardenensis* (NRRL Y-17526), *Brettanomyces custersianus* (NRRL Y-6653), *Brettanomyces nanus* (NRRL Y-17527), *Hanseniaspora uvarum* (NRRL Y-1614), *Hanseniaspora guilliermondii* (NRRL Y-1625), *Hanseniaspora occidentalis* (NRRL Y-7946), *Hanseniaspora osmophila* (NRRL Y-1613), *Hanseniaspora valbyensis* (NRRL Y-1626), *Hanseniaspora vineae* (NRRL Y-17529), *Candida stellata* (NRRL Y-1446), *Kloeckera lindneri* (NRRL Y-17531), *Saccharomyces cerevisiae* (ATCC 4098), *Saccharomyces kluyveri* (ATCC), *Torulaspora delbrueckii* (NRRL Y-866), *Zygosaccharomyces bailii* (ATCC 66825), *Zygosaccharomyces rouxii* (NRRL Y-229), *Zygosaccharomyces bisporus* (NRRL Y-12626).

Selection of PNA probe sequences

Sequence analyses were performed using computer software from DNASTAR (Madison, WI, USA) and by 'Advanced BLAST' searches of the GeneBank nr-database. Alignments

of closely-related rRNA sequences obtained from the GeneBank database were done using the Megalign (v. 4·03) software (Boston Probes Inc, MA, USA). From such alignments of the different rRNA sequences, potential PNA probes (typically 15-mers) specific for individual bacteria or yeasts were identified. Lack of any significant level of secondary structure of the complementary PNA probe sequences were checked using the PrimerSelect software (v. 4·03) (Boston Probes Inc, MA, USA). Finally, remaining probes were checked for any significant sequence similarity against the whole GeneBank database using the GeneMan (v. 3·30) software (Boston Probes Inc, MA, USA) and by an Advanced BLAST search of the GeneBank nr-database (HTTP://www.ncbi.nlm.nih.gov\blast).

In addition, two universal probes were constructed. BacUni-1 was a modified PNA version of the universal eubacterial DNA probe (EUB338) described by Amann *et al.* (1990). The EuUni-1 probe was a modified PNA version of the eucarya domain probe described by Amann *et al.* (1995).

Synthesis of the peroxidase-labelled PNA probes

The PNA probe sequences used in this study are listed in Tables 1 and 2. PNA probes were synthesized in-house using commercially-available reagents and instrumentation (PE Biosystems, Foster City, CA, USA). Synthesis and purification of the probes was improved using PNA solubility enhancers (Gildea *et al.* 1998). Probes were purified by HPLC and their integrity and purity were verified by mass spectrometry. Lyophilized probes were resuspended in 50% aqueous DMF and their concentration was verified by measuring u.v. absorption at 260 nm (Nielsen and Egholm 1999). The probes were then conjugated to soybean peroxidase using a recently published procedure (Coull and Fitzpatrick 1999) and purified by size exclusion chromatography.

Dot blot hybridization

Cells from exponentially-grown cultures of the bacteria listed in Table 3 were harvested by centrifugation, and total RNA was purified following the protocol given in a commercially-available kit (Qiagen, Valencia, CA, USA). The quantity of isolated RNA was determined by measuring the absorbance at 260 nm. Total RNA (20 μg ml^{-1}) isolated from the different micro-organisms was diluted to 20 ng ml^{-1} using DEPC (D5758, Sigma)-treated water. Each dilution was heated to 68°C for 3 min to denature the RNA before spotting 1 μl of solution onto a non-charged nylon membrane (Biodyne A; Gibco/BRL, Rockville, MD, USA). The spotted RNA dots were allowed to dry at room temperature before the RNA was cross-linked by exposing the membrane to 33 000 μjoules of u.v. irradiation using a Stratalinker (Stratagene, La Jolla, CA).

Table 1 Results of dot blot hybridization. Nine SBP-labelled PNA probes were analysed using purified RNA from 10 different bacterial species and one yeast species

Probe name	Target/s	Sequence (5'-3')	*Ps. fluorescens*	*Ps. aeruginosa*	*B. cepacia*	*Ps. putida*	*E. coli*	*B. subtilis*	*Staph. epidermidis*	*Staph. aureus*	*Salm. choleraesuis*	*Lact. brevis*	*S. cerevisiae*
BacUni-1	Eubacteria	CTG CCT CCC GTA GGA	+	+	+	+	+	+	+	+	+	+	+
EuUni-1	Eucarya	ACC AGA CTT GCC CTC	−	−	−	−	−	−	−	−	−	−	−
Eco16S06	*E. coli*	TCA ATG AGC AAA GGT	−	−	−	−	+	−	−	−	+	−	−
Sal23S15	*Salm.*	ACC TAC GTG TCA GCG	−	−	−	−	−	−	−	+	−	−	−
Sta16S03	*Staph. aureus*	GCT TCT CGT CCG TTC	−	−	−	−	−	−	−	−	−	−	−
Pse16S32	*Ps. aeruginosa*	CTG AAT CCA GGA GCA	−	+	−	−	−	−	−	−	−	+	−
Lac16S08	*Lact. brevis*	CTC TAA GAT TGG CAG	−	−	−	−	−	−	−	−	−	−	+
Sce18S07	*S. cerevisiae*	TTA CCG AGG CAA GCT	−	−	−	−	−	−	−	−	−	−	−
Zba18S03	*Z. bailii*	CGA GCG AAA CGC CTG	−	−	−	−	−	−	−	−	−	−	−

Table 2 Results of dot blot hybridization. Six different SBP-labelled PNA probes were analysed using purified RNA from fifteen different yeast species and one bacterial species

Probe name	Target/s	Sequence (5'-3')	*D. bruxellensis*	*D. anomala*	*B. naardenensis*	*B. custersianus*	*B. nanus*	*H. uvarum*	*H. guilliermondii*	*C. stellata*	*K. lindneri*	*T. delbrueckii*	*Z. bailii*	*Z. rouxii*	*Z. bisporus*	*S. kluyveri*	*S. cerevisiae*	*Lact. brevis*
BacUni-1	Eubacteria	CTG CCT CCC GTA GGA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
EuUni-1	Eucarya	ACC AGA CTT GCC CTC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Sce18S07	*S. cerevisiae*	TTA CCG AGG CAA GCT	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
Bre26S14	*D. bruxellensis*	CGG TCT CCA GCG ATT	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Zba18S03	*Z. bailii*	CGA GCG AAA CGC CTG	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−
Lac16S08	*L. brevis*	CTC TAA GAT TGG CAG	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

Table 3 Growth conditions for micro-organisms used in this study for both traditional colony counts and for the membrane *in situ* hybridization assay using PNA probes. For each micro-organism, the growth medium and temperature were the same, whereas the incubation time varied between the two assays

Organisms	Medium	Temperature (°C)	Time (h)	
			Colonies	PNA assay
E. coli	LxB	35	16	4
Ps. aeruginosa	TSA	35	18	5
Ps. fluorescens	TSA	30	18	5
Salm. choleraesuis	TSA	35	16	5
Staph. aureus	TSA	30	48	15
*Lact. brevis**	TJA	30	240	48
S. cerevisiae	YM	30	24	12
D. bruxellensis	BSM	30	240	40
Z. bailii	YM	30	48	15

* Grown under anaerobic conditions.

Each membrane was then placed in a plastic bag and pre-hybridized for 15 min at 50°C in 1 ml hybridization buffer 20 cm^{-2} membrane. The hybridization buffer (HB) consisted of 25 mmol l^{-1} Tris–HCl, pH 9·5, 50% formamide, 0·7% Tween, 1% DEPC-treated casein, 100 mmol l^{-1} NaCl, 1× Denhardts solution and 2% polyvinylpyrrolidone. The HB was removed and fresh HB (0·1 ml HB cm^{-2} membrane) containing the SBP-labelled PNA (1 pmol ml^{-1}) was added to the bag. The blot was hybridized at 50°C for 30 min and excess probe was removed by washing the membrane three times in 100 ml Wash Buffer (10 mmol l^{-1} Capso, pH 10; 0·2% Tween 20). The first wash was performed at room temperature for 5 min whereas the last two washes were conducted at 50°C for 15 min each.

After the washes were completed, the membrane was incubated in chemiluminescent substrate (0·2 ml cm^{-2} of membrane; SuperSignal, Pierce, Rockford, IL, USA). The membrane was placed in a small plastic bag and excess substrate was squeezed out of the bag before it was sealed and exposed to X-ray or instant film for 1–10 min.

Detection of organisms on filters

Filtration. Diluted cultures, or samples containing micro-organisms, were filtered through 47 mm, 0·45 μm pore size membranes (type HVLP, Millipore, Bedford, MA, USA). Individual membranes were then placed on either medium-soaked cellulose pads in Petri dishes or agar medium, and incubated for various times.

The types of media used included Lennox Broth (LxB, Sigma), Tryptic Soy Agar (TSA, VWR, Bridgeport, NJ, USA), Tomato Juice Agar (TJA, VWR), Yeast and Mold Medium (YM,VWR) and Brettanomyces Specific Medium

(BSM, Millipore). The type of medium used was dependent on the micro-organism that was being tested (see Table 3).

Incubation. The membranes were incubated at optimal growth temperature for approximately one third of the time required for visible colonies to form (see Table 3).

Fixation. The membranes were removed from the media and cells were fixed by placing each membrane face up on a cellulose pad that was soaked with fixative (95% ethanol; 0·35% glutaraldehyde; 0·03% (w/v) urea-hydrogen peroxide; and 5 mmol l^{-1} sodium azide). The fixation solution was formulated to kill, fix and cross-link the cells to the membrane, as well as inhibit endogenous peroxidase activity (Li *et al.* 1987).

Hybridization. After 5 min of exposure to fixative, each membrane was moved to a Petri slide (Millipore) containing 1·5 ml HB and 5 pmol ml^{-1} SBP-conjugated PNA probe. The filters were then hybridized in an incubator at 50°C for 30 min. Doubling the probe concentration to 10 pmol ml^{-1} increased the signal but in some cases, also led to cross reactivity (data not shown).

Washing. The hybridization buffer was poured off, and the membranes were washed for 4 × 7 min at 50°C with pre-warmed Wash Buffer (10 mmol l^{-1} Capso, pH 10; 0·2% Tween 20) using a specially designed eight membrane wash rack (Boston Probes, Bedford, MA, USA).

Detection. The membranes were then transferred to 0·5 ml chemiluminescent substrate (Pierce No. 17015 prepared according to the manufacturer's directions) and incubated for 2 min at room temperature. Following substrate incubation, membranes were transferred to heat-sealable bags, excess substrate was squeezed out and the bags were sealed.

Visualization. The membranes were exposed for 15 min to X-ray film (Fuji RX, E.M. Parker, Wilmington, MA, USA) in a film cassette, or 20 000 ASA instant film (Boston Probes) using a Spotlight Camera (Boston Probes).

RESULTS

Evaluation of enzyme-labelled PNA probes

Filtration followed by incubation of membranes on growth media from 18 h up to several weeks is a standardized microbiological procedure (Anon. 2000). In order to achieve a more rapid and sensitive assay procedure, rRNA reactive PNA probes were conjugated directly to soybean peroxidase (SBP) for use with a chemiluminescent substrate.

The SBP-labelled PNA probes were tested for specificity, sensitivity and background using dot blots with immobilized RNA. Figure 1 illustrates typical results from such probe screening experiments. In this figure, two sets of RNA blots, comprising nine purified bacterial RNAs and nine purified yeast RNAs, were probed with the various SBP-labelled PNA probes indicated. The SBP-labelled species-specific probes reacted strongly with the intended micro-organisms, and the universal bacterial probe (BacUni-1) reacted strongly with all bacterial rRNAs tested but did not hybridize to RNA isolated from any of the different eukaryotic yeast cells. Conversely, the SBP-labelled

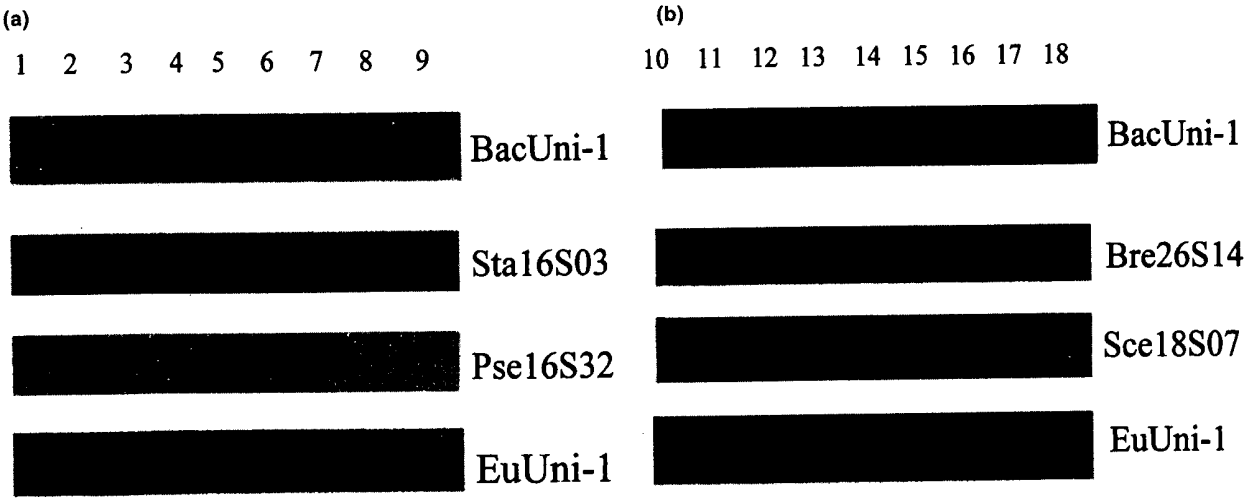

Fig. 1 X-ray images from evaluation of SBP-labelled PNA probes by dot blot hybridization using RNA from (a) nine bacterial species (1: *Pseudomonas fluorescence*, 2: *Ps. aeruginosa*, 3: *Burkholderia cepacia*, 4: *Ps. putida*, 5: *Escherichia coli*, 6: *Bacillus subtilis*, 7: *Staphylococcus epidermidis*, 8: *Staph. aureus*, 9: *Salmonella choleraesuis*) and from (b) eight yeast species (10: *Candida stellata*, 11: *Dekkera bruxellensis*, 12: *Hanseniaspora guilliermondii*, 13: *Kloeckera lindneri*, 14: *Zygosaccharomyces bailii*, 15: *H. uvarum*, 16: *Saccharomyces kluyveri*, 17: *Z. rouxii*, 18: *S. cerevisiae*)

Table 4 Correlation of the number of visible colonies following sustained growth to the number of colony-forming units determined by spots using the membrane *in situ* hybridization PNA assay

Organisms	Visible colonies (mean ± S.D.)	Spots (mean ± S.D.)	Correlation (%)
E. coli	41·0 ± 6·2	40·6 ± 6·4	98·9
Ps. aeruginosa	86·8 ± 8·1	83·3 ± 10·2	95·6
Staph. aureus	32·6 ± 6·6	32·5 ± 6·4	99·8

universal eukaryotic probe (EuUni-1) hybridized strongly to all nine yeast RNA preparations and not to any of the nine bacterial RNA preparations. These two probes also served as positive controls to ensure the presence of bacterial and eukaryotic RNA on the blots. Finally, none of the probes gave rise to any significant background due to general non-specific binding of the SBP-conjugated probes to the membranes.

Table 1 shows the sequences of the different bacterial PNA probes examined and summarizes their dot blot specificity. All of the bacterial probes hybridized with a high degree of specificity. Table 2 shows the different yeast PNA probes as well as the dot blot specificity results for these probes. The *D. bruxellensis* PNA probe (Bre26S14) was very specific while the *S. cerevisiae* PNA probe (Sce18S07) cross-reacted with *T. delbruckii*. Additionaly, the *Z. bailii* PNA probe (Zba 18S03) cross-reacted significantly with both *C. stellata* and *Z. bisporus*. Although reactivity with the *Z. bisporus* strain was expected because of known sequence homology, the reactivity with *C. stellata* was surprising. Subsequent database searches, which included new sequence information, revealed that this target sequence was found in several different *Candida* strains.

Membrane *in situ* hybridization

Probes passing the dot blot evaluation were further tested in a rapid membrane-based assay. Bacteria and yeast species were filtered and incubated for various times on cellulose pads impregnated with growth media. After removal from the media pads, filters were fixed and hybridized as described in the Materials and Methods.

Figure 2 shows the results obtained from the filtration of *E. coli* followed by probing with either BacUni-1 or EuUni-1. With the BacUni-1 probe, *E. coli* microcolonies were visible after only 90 min of growth (Fig. 2a). The signal strength clearly increased as the microcolonies were grown for longer periods of time (Fig. 2b, c). Comparison of spot counts for the various time points revealed that approximately 90% of the total number of colonies were visible after the initial 90 min of growth. No signal was seen when the eukaryotic universal probe (EuUni-1) was substituted for BacUni-1. Both Gram-negative and Gram-positive bacteria were tested in this assay, with similar results (Table 3). The length of the growth period that was required before colonies were detected using the PNA assay varied widely dependent on the bacteria or yeast strain used, but in all cases was significantly less (approximately 1/3) than the growth time needed to obtain visible colonies.

During the fixation, cells were attached to the membrane surface and the rRNA inside each cell was made accessible to the SBP-labelled PNA probe. The preserved cell morphology was verified by analysing membranes that had been hybridized with fluorescein-labelled probes instead of SBP-labelled probes. The membranes were mounted on slides under a coverslip and visualized using a fluorescent microscope. The majority of the cells were found to be intact and brightly fluorescent as shown in Fig. 3.

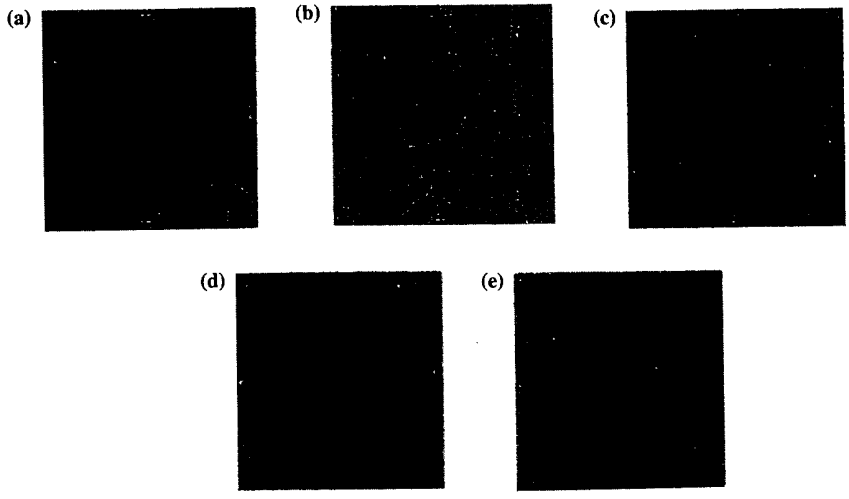

Fig. 2 X-ray images of *Escherichia coli* analysed by membrane *in situ* hybridization following growth on LxB at 35 °C for (a) 90 min, (b) 135 min, and (c–e) 195 min. Hybridization was performed with (a–c) BacUni-1, (d) EuUni-1 and (e) no probe

Enumeration

In order to correlate spots on the X-ray film to the number of colony-forming units (cfu), overnight cultures of *E. coli*, *Ps. aeruginosa* and *Staph. aureus* were diluted in PBS prior to filtration. Forty-eight identical membranes were prepared for each micro-organism and these were incubated on growth media as indicated in Table 4. Half of the membranes were incubated until colonies were visible, typically 16–36 h, and the number of colonies on each membrane was counted. The other half of the membranes were fixed and assayed after 4 h and 30 min (*E. coli*), 5 h (*Ps. aeruginosa*) and 15 h (*Staph. aureus*) of growth. As shown in Table 4, there was excellent correlation (>95%) between the number of colonies which grew to visible size on the membranes and the number of spots seen on the X-ray film.

Identification

The assay principle was tested on different human pathogens, environmental contaminants and spoilage organisms, including Gram-negative and Gram-positive bacteria as well as different yeasts. Figure 4 shows X-rays of filters on which a suspension of the Gram-positive bacterium *Staph. aureus* was filtered, incubated for 15 h at 30°C, and probed with either EuUni-1, BacUni-1 or Sta16S03. The *Staph. aureus*-specific PNA probe (Sta16S03) and the universal eubacteria probe (BacUni-1) both hybridized strongly to the micro-colonies on the membranes, while the universal eucarya probe (EuUni-1) did not.

Figure 5 illustrates results of membrane experiments where suspensions of *E. coli* or *Ps. aeruginosa*, as well as mixtures of both, were filtered and grown on TSA at 35°C for 5 h. Following fixation, membranes were hybridized with either the *E. coli*-specific probe, Eco16S06, the *Ps. aeruginosa*-specific probe, Pse16S32, or the universal eubacteria probe, BacUni-1. The results clearly demonstrated the ability of the two species-specific probes to distinguish between the different bacterial types, as well as the ability of the BacUni-1 probe to detect both types of bacteria. The *Ps. aeruginosa* membrane was blank when probed with the *E. coli*-specific probe, and the membrane with *E. coli* was blank when probed with the *Ps. aeruginosa*-specific probe. Moreover, it was found that if the membranes were kept wet after film exposure, they could be reprobed using a different probe. This allowed the successive detection of different species on the same membrane.

Fig. 3 Microscope image of an *Escherichia coli* microcolony. *Escherichia coli* diluted in PBS was spotted onto a membrane, grown on LxB at 35 °C for 5 h, analysed by the membrane *in situ* hybridization method using a mixture of fluorescein-labelled BacUni-1 and Eco16S06, each at 5 pmol ml^{-1}, and subsequently examined by fluorescence microscopy

Use of instant film

In order to compare results from instant film and X-ray film, *E. coli* cells were filtered and grown on LxB media at 35°C for 4 h. Following fixation, the filters were hybridized with the *E. coli*-specific probe (Eco16S06), washed, and placed in chemiluminescent substrate. Figure 6 shows a comparison of the results obtained using either a Spotlight camera box loaded with 20 000 ASA instant film, or X-ray film developed with an automated processor. Virtually identical results were obtained with the two types of film.

DISCUSSION

Membrane filtration is commonly used for collection of micro-organisms from many different types of samples,

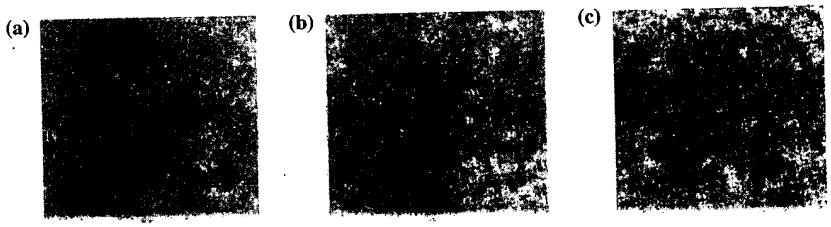

Fig. 4 X-ray images of *Staphylococcus aureus* analysed by membrane *in situ* hybridization following growth on TSA at 30°C for 15 h. Hybridization was performed with (a) EuUni-1, (b) Sta16S03 and (c) BacUni-1

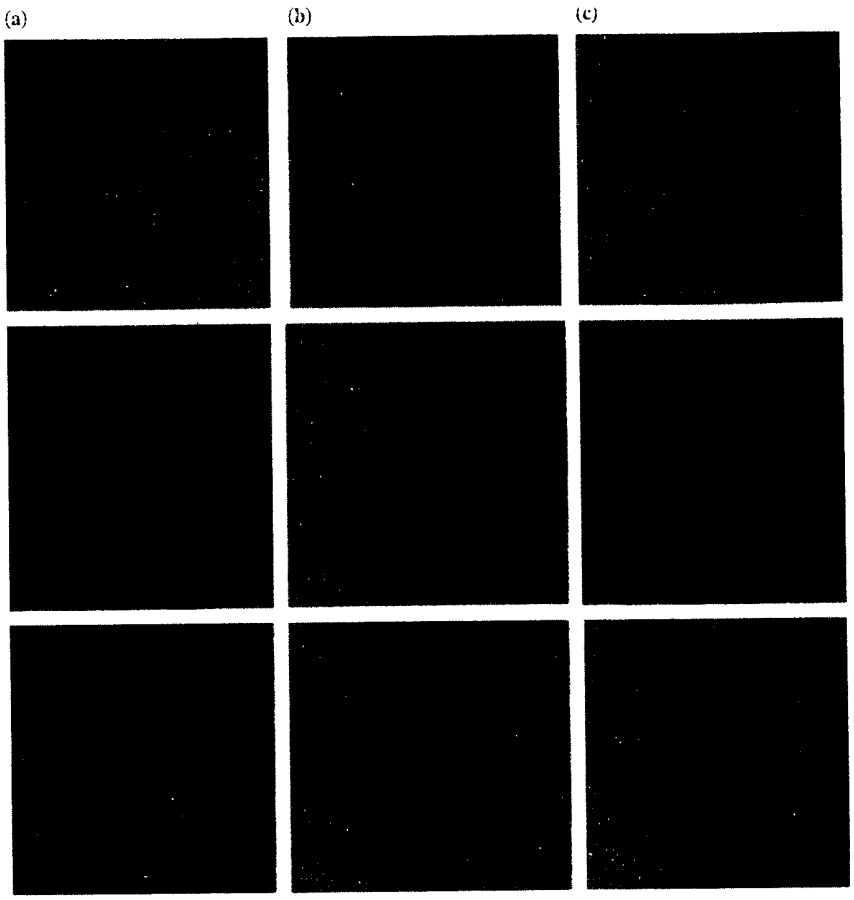

Fig. 5 X-ray images of (a) *Escherichia coli*, (b) a mixture of *E. coli* and *Pseudomonas aeruginosa*, and (c) *Ps. aeruginosa*, respectively, analysed by membrane *in situ* hybridization. Hybridization was performed using Eco16S06 (top row), BacUni-1 (middle row) and Pse16S32 (bottom row) as indicated above

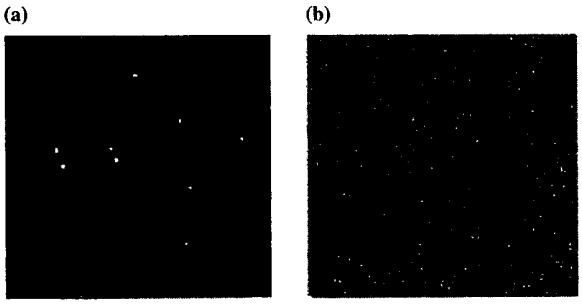

Fig. 6 Images of (a) instant film and (b) X-ray film of *Escherichia coli* analysed by membrane *in situ* hybridization. Hybridization was performed using Eco16S06, as indicated above. The membrane was exposed to both instant film and X-ray film for 15 min for direct comparison

including liquids and gases. Over the years, a number of validated applications of the filter technique have been described, including sterility, bioburden and limits testing (Anon. 1999; Anon. 2000). It is estimated that more than 100 million membrane assays are performed annually worldwide (Dr Molly Pickett, Millipore Corporation, personal communication). One of the primary advantages of the membrane method relates to the ability to test large sample volumes instead of being restricted by the volume that can be spread on an agar plate. As more rapid procedures allow for earlier detection, diagnosis and corrective action, much effort has been aimed at reducing the time-to-result of the membrane filter technique. One example is the continued development of improved media for fast recovery and growth of stressed organisms (McFeters 1982). Another example is the development of new instrument platforms like the MicroStar System (Millipore) which uses ATP-bioluminescence and a camera-type instrument. The MicroStar platform is capable of detecting bacterial microcolonies of only a few dozen living cells on the membrane surface after just a few hours of growth (Tanaka *et al.* 1997; Takahashi *et al.* 1999). However, as all living organisms contain ATP, the identity of the

organism is only as certain as the selectivity of the media used for the growth step.

It was believed that combining a direct enzyme-labelled PNA probe with a chemiluminescent based-detection system would provide results as rapidly as membrane-based ATP-bioluminescence with the added certainty of probe-based identification. The results presented here demonstrate rapid detection, identification and enumeration of Gram-negative and Gram-positive bacteria, as well as yeast, using a single protocol and various peroxidase-labelled PNA probes. To date, the method has been successfully extended to over 50 different micro-organisms grown on a variety of media without the need to adjust or optimize the fixation, hybridization or detection steps of the protocol. Typically, micro-organisms were detected in less than one-third the growth time required to see colonies by eye. It is believed that the generality of the method can be ascribed to the better hybridization properties of PNA probes for targeting structured molecules such as rRNA (Stefano and Hyldig-Nielsen 1997). This is, as far as is known, the first example of the use of enzyme-labelled probes for whole cell *in situ* hybridization of Gram-positive bacteria and yeasts.

The sensitivity of this assay method was not sufficient to detect single bacteria or yeast cells directly after filtration but required a short growth step. This ensured that only viable and culturable cells were detected, while neither the so-called viable non-culturable nor dead micro-organisms gave rise to a signal. It was also found that it was impossible to circumvent the growth step by spotting a large number ($<10^5$ μl^{-1}) of bacteria on a membrane (data not shown). This indicates that the growth step either ensures a better fixation of the cells to the membrane, or that only a true microcolony can emit sufficient light to create a signal on the film. If a growth rate of 30 min for a rapidly-growing bacterium such as *E. coli* is assumed, it can be calculated that the assay can detect a microcolony consisting of less than 12 cells (90 min post-filtration). These last points are important, as whole-cell PCR and other amplification methods based on purified nucleic acids may detect dead cells present in a sample. Such cells are usually of no concern to the industrial or clinical microbiologist.

As this method uses a standard membrane filter and is compatible with a wide variety of general and highly selective media, it is believed that it could be useful for rapid enumeration and identification of micro-organisms. Also, excellent correlation to plate counts for both Gram-negative and Gram-positive bacteria has been demonstrated, indicating that the PNA whole-cell hybridization assay is highly analogous to a standard plate count but with the added features of a faster assay and simultaneous identification of specific organisms of interest. However, lower correlations were experienced with slow-growing yeast species such as *D. bruxellenis* and *Z. bailii* (data not shown). The reasons for this are not yet clear and require further study. In addition, it was found that it was possible to reprobe the filters, without stripping them, if a different site on the rRNA was targeted. This allows multiple assays to be performed sequentially on the same filter.

The light emitted from the membranes was captured on instant film or X-ray film. The ability to use a hand-held Spotlight camera box and instant film provides portability and allows data to be acquired when a dark room facility is not available. Alternatively, X-ray film allows many filters to be imaged simultaneously, providing higher throughput. It is likely that the light emitted from the membrane can be digitally captured using a CCD camera or other digital systems such as the Microstar System. This is currently under investigation.

In all cases examined, the rapid filter assay described here significantly shortened the time required for detection, enumeration and identification of micro-organisms compared with standard growth and counting methods, because the filter assay allowed results to be obtained well before colonies were visible on the membranes. For organisms such as *E. coli* and *Ps. aeruginosa* that can be recovered rapidly with 4–5 h of growth, results may be obtained in less than one working day.

This new assay format may be used for a variety of different applications within clinical, industrial and environmental microbiology. This will be determined to a large extent by the specificity of the PNA probe, which may range from kingdom- to species-specific. For example, a species-specific PNA probe might be used for quality control of consumer-related products that must be declared free of a certain pathogen, spoilage or indicator organism prior to release, while a universal probe might be used for sterility testing.

The technology described here is currently being extended to specific applications in industrial microbiology, such as rapid testing of municipal and bottled water (Stender *et al.* 2000). Future work will also include expansion of the method for detection of fungi and moulds.

ACKNOWLEDGEMENTS

The authors would like to thank Shelagh Casey, Susan Voetsch and Joan MacNeill for preparation and purification of the PNA probes used in this study. They greatly appreciate technical and financial support given by Millipore Corporation.

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